

AFFECTS OF MEDIA AND MEDIA AMENDMENTS ON  
THE IN VITRO CULTURE OF PUCCINIA  
RECONDITA F. SP. TRITICI

By

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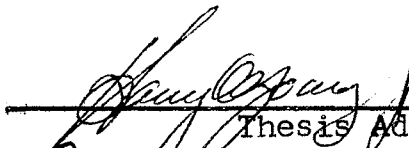
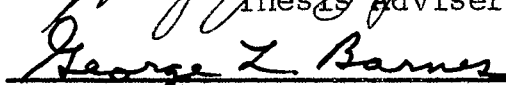
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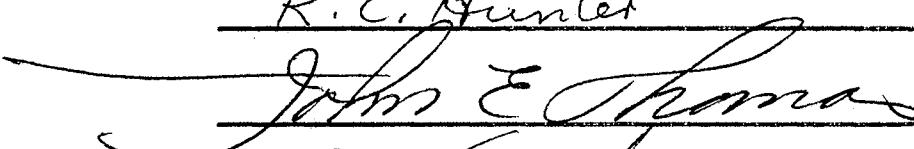
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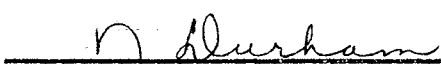
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## CHAPTER I

### INTRODUCTION

As a group of plant-disease-causing organisms, the rust fungi are undoubtedly among the most economically important. One has only to reckon the cost of the wheat stem rust outbreak in 1935 in the United States or the 1878 coffee rust epidemic in Ceylon, to appreciate the destructiveness of this group of fungi (12, 22).

Among the fungus plant pathogens, the Uredinales or rust fungi, usually have been referred to as classical examples of obligate parasites. Although they still cannot be routinely grown in artificial media the successful growth of some species in the absence of tissues of their specific hosts has been reported. However, examples are limited and in most cases special techniques were necessary before growth could be obtained.

The cultivation in vitro of the stem rust fungus, Puccinia graminis Pers. f. sp. tritici (Eriks. & E. Henn.) Australian race 126 ANZ 6,7, by Williams et. al. (23, 24), and confirmation of their results by Bushnell (4) seems to have given impetus to research about the culturing of the rust fungi. As a subject of research it appears to be gaining the interest of a steadily increasing number of investiga-

tors. The reasons for this intensified interest are many and varied. However, perhaps the most explicit reason was stated in a 1951 editorial in Nature (1) which emphasized the significance of cultivating pathogens free of their hosts or other organisms. It stated that " When we know how the parasite breathes, feeds, excretes and uses, within its host and at its host's expense, whatever biochemical processes link it to that host, we may learn to control it. "

It is probable that exchange of nutrients or toxins or both, between the rust fungus and host may determine infection type exhibited by the disease. Axenic cultural studies could prove very useful in investigating these interactions and therefore help in identifying the mechanisms that determine host susceptibility or resistance.

The objective of this study was to examine the effects of various media and media amendments on growth and sporulation of one of the obligate parasites. The fungus P. recondita f. sp. tritici was chosen for the study because initial work on its axenic culture had been done at this institution and because, in nature, this parasite is one of the most economically important in this area.

## CHAPTER II

### REVIEW OF LITERATURE

One of the early reports about growth of a rust fungus on an artificial medium is that of Hotson and Cutter (13). They observed mycelia of Gymnosporangium juniperi-virginianae Schw. growing out from callus tissue and invading the nutrient substrate on which the tissues were being cultivated. They further noted that the fungus showed abortive attempts to sporulate when placed on a number of synthetic media.

Cutter (11) reported saprophytic growth of P. malvacearum on agar medium. Telial pustules with mature teliospores were observed in three-month old callus tissues. In one instance, a minute colony was noted on the agar surface a few mm from the base of the callus stem. Cutter surmised that the colony had originated from the germination of a basidiospore or directly from the promycelium of a teliospore, which had fallen on the agar surface since no visible connection to the stem was noted.

Attempts to obtain axenic cultures of other rust fungi are continuing. One species whose saprophytic growth is being extensively studied is P. graminis f. sp. tritici. Vegetative growth of an Australian isolate of this fungus

was described by Williams et. al. (23) in 1966. A year later, the same isolate was found to sporulate on a medium containing Czapek's minerals, sucrose and agar (24).

Initial tests to grow North American isolates of the wheat stem rust fungus in artificial medium failed (4). In a later series of tests, however, Bushnell and Stewart (6) succeeded in growing 12 out of 16 races. Some of these had failed to grow during earlier experiments. Macko et. al. (16) obtained rather limited saprophytic growth of P. graminis tritici, race 56.

The following growth characteristics were described by Kuhl et. al. (15) when they inoculated uredospores of various Puccinia spp. on artificial media : profuse mycelia formation of certain races of P. graminis avenae, fair mycelia formation of certain races of P. graminis secalis and P. coronata avenae. With cultures of P. sorghi, they noted sparsely branched hyphae which did not grow beyond the sporeling stage.

The ability of other species to grow saprophytically also have been reported. In their work on P. recondita f. sp. tritici, the wheat leaf rust pathogen, Singleton and Young (20) observed vegetative growth on a solid medium containing Evan's peptone, Czapek's Dox Broth and yeast extract.

Reports of saprophytic development have not been limited to the Pucciniaceae. Axenic growth of the Melampsoraceae have likewise been obtained. Turel (21) reported the

results of her work on the saprophytic development of the flax rust fungus, Melampsora lini, race 3. She claimed that the fungus produced clusters of uredospore-like bodies from dense masses of firm cells formed on artificial medium. She added that aerial hyphae yielded one-celled teliospores. Coffey et. al. (8) confirmed Turel's findings. They also noted spore-like cells of varying shapes some of which resembled uredospores and teliospores.

Differences in opinion have been expressed regarding the prerequisites for saprophytic growth by the rust fungi. Failure of earlier attempts to obtain axenic cultures has led to the idea that infection structures, as formed in host plants, may be important prerequisites for saprophytic development. For instance, Brown (3) suggested that much of the nutritional specificity of the obligate parasites might disappear if formation of haustoria could be induced under artificial conditions.

The positive role of infection structures for saprophytic growth became even more convincing when Hurd-Karrer and Rodenheiser (14) reported that primary and secondary vesicles were formed when uredospores of six Puccinia species, excluding P. recondita f. sp. tritici, were inoculated on artificial medium. They further reported that septate infection hyphae up to 300  $\mu$  long developed from secondary vesicles.

However, it is now obvious that infection structures like those produced in host infections, are not prerequi-

sites for growing the rust fungi saprophytically. Rather, the problem seems to be one of finding the correct nutrients. This opinion is supported by the results of recent work dealing with this particular subject (5, 7).

## CHAPTER III

### MATERIALS AND METHODS

#### Races

Eight of the most common local races of the leaf rust fungus, *P. recondita* f. sp. *tritici*, were tested for their ability to grow axenically in artificial media. These races (UN 1A, UN 2, UN 2A, UN 2AAG, UN 5A, UN 6B, UN 9 and UN 13) were identified by their virulence on the "Unified Numeration" set of differential wheat cultivars established by Basile (2). Additional virulence is designated by a letter or letters following the race number (A - virulence on the cultivar Westar CI 12110; B - virulence on both Westar and Wesel CI 13090; AG - virulence on the cultivar Agent CI 13523). An isolate of *P. graminis* f. sp. *tritici* from Australia (Race 126 ANZ 6,7) obtained from the American Type Culture Collection, Culture PR-48), Rockville, Md. was also used in certain studies.

#### Sterile Uredospores

Inocula used to obtain axenic growth were uredospores since they are prolifically and uniformly produced under carefully controlled conditions. The use of a method that

ensures the production of sterile uredospores cannot be overemphasized. A supply of uncontaminated uredospores is indispensable when studying their behavior on artificial media.

The technique followed to obtain sterile uredospores was a modification of that used by Singleton and Young (20). Six-day old plants of the wheat cultivar Cheyenne CI 8885 were placed inside a moist chamber and sprayed with a solution containing tap water and a surfactant, Tween 20 (Polyoxyethylene 20 sorbitan monolaurate) at 3-4 drops/1000 ml water. The plants were inoculated by brushing with the leaves of a plant infected with the desired race of the fungus. The plants were sprayed again and left overnight in the moist chamber. The following day the plants were transferred to the greenhouse for disease development. Five to six days after inoculation, when the leaves showed distinct and well-developed flecks, the infected plants were brought inside the laboratory where the most heavily flecked leaves were detached at the base and suspended in sterile-distilled water in Petri dishes.

Prior to placing the detached leaves on a medium which would support the leaf until uredospores were formed, it was necessary to sterilize the leaf surface so that the uredospores used for inoculum would be free of contaminants. At first, the leaves were surface-sterilized by suspending them for three minutes in a solution of 5.25% sodium hypochlorite and Tween 20 (3-4 drops/1000 ml sodium hypochlo-

rite solution). It was observed however that this procedure was not satisfactory for destroying certain contaminants, especially bacteria. In some cases as many as 80% of the leaves were not rendered surface-sterile, as shown by the appearance of fast-growing bacterial colonies that contaminated the entire Petri dish even before the uredospores appeared.

It was therefore necessary to find a better technique for surface sterilizing flecked leaves. Consequently, four dilutions of mercuric bichloride (0.5, 1.0, 1.5 and 2.0 gm/1000 ml water) were prepared and used as surface-sterilants for the flecked leaves. Eight to ten leaves were dipped instantaneously into 75% ethyl alcohol prior to surface sterilization to reduce surface tension. Various lengths of treatment in the mercuric bichloride solutions, ranging from 30 seconds to three minutes were tried.

A modification of the above method was tried. This modification consisted of suspending the leaves directly in the mercuric bichloride solution to which Tween 20 was added (1.5 ml Tween 20/1000 ml mercuric bichloride solution) to reduce surface tension. It was thought that sterilization of the leaves could be done in this manner without the alcohol treatment.

The primary considerations in deciding the most effective dilution and length of treatment with the mercuric bichloride solutions were: freedom from contaminants and the absence of scalding, burning, rolling or any sign of phyto-

toxicity on the treated leaves. The results indicated that suspending the leaves for one minute in a solution containing 1.5 gm mercuric bichloride and 1000 ml distilled water was most effective in surface-sterilizing the leaves. The leaves that were given this treatment were practically free of contaminants and did not show any phytotoxic effect. They remained green sufficiently long for the production of contaminant-free uredospores.

Dipping the leaves in ethyl alcohol prior to treatment with the mercuric bichloride solution was found to be necessary. This treatment was used throughout the study and served to insure thorough wetting of the leaves with the mercuric bichloride solution. This method was at least 50% more effective than the use of Tween 20 in the mercuric bichloride solution.

Surface-sterilized leaves were rinsed in three changes of sterile-glass-distilled water and then incubated on congealed media in Petri dishes. Three to four leaves were arranged side by side with the abaxial side in contact with the medium. The excised end of the leaves were buried slightly into the medium. Two kinds of media, potato-dextrose agar (PDA) and water agar (WA) were used. PDA, as a medium for incubating surface-sterile-flecked leaves for the purpose of yielding spores, proved superior to water agar since the growth of contaminants could be detected more readily and at an earlier time.

The leaves on these media were kept inside an incubator (Percival Model E-30) maintained at  $17 \pm 1$  C. Continuous light (5381.95 lux) was provided until the uredosori broke through the epidermis and uredospores were released.

### Test Media

Four basal media were tested, (Appendix, Table I). Medium I was simply PDA used commonly for fungus culture. Media II and IV were previously reported to support, to some extent saprophytic growth of certain species of rust fungi (20, 21). Medium III was a slight modification of that used by Coffey *et. al.* (7) in their study of the culture of the stem rust fungus.

The general procedure for the preparation of each of the media was as follows. The ingredients were weighed, added separately to the desired volume of glass-distilled water and thoroughly stirred with a glass rod. Whenever necessary the solution was heated in the autoclave to dissolve ingredients that were not readily soluble in cold water. The pH was adjusted to 6.0 after all the ingredients had been completely dissolved. A final check of the pH was made after the medium was sterilized in the autoclave at 121 C for 20 minutes and cooled to approximately 80 C. A sterile pipette was used to add the necessary amount of 1 N HCl or NaOH to obtain the desired pH. The pH was measured with a Beckman Zeromatic pH meter. Usually, two to three liters of each medium were prepared in bulk and stored at 12 to 15 C.

### Inoculation

The test media were seeded with sterile uredospores using three methods. The first method consisted of picking up the sterile spores from the detached leaves with a cotton swab and transferring them to the test media. The spores were deposited by touching or lightly brushing the cotton swab on the surface of the medium. In the second method the leaf containing the uredospores was lifted carefully from the medium surface with a sterile forceps, held slightly above the surface of the media to be seeded and tapped gently to dislodge the uredospores. In the third inoculation method, a 15% gelatin suspension was prepared and sterilized in the autoclave at 121 C for 20 minutes. Uredospores were added to the gelatin suspension and small drops of the suspension, 3-5 mm in diameter, were placed on the congealed medium.

The second method consisting of seeding the growth media by allowing the spores to drop freely to the medium was observed to be decidedly more satisfactory than the other two methods. In general, initiation of growth occurred earlier and progressed faster with this method. Quite frequently, spores deposited with a cotton swab (first method) showed no sign of germination when those seeded by allowing the spores to drop freely to the medium were already growing.

### Handling Procedures

All seeded media were incubated without light in an incubator (Percival Model E-30) at  $17 \pm 1$  C. When Petri dishes were used they were sealed with masking tape to minimize contamination from outside and to retard drying of the medium. When flasks were used they were plugged with cotton prior to sterilization. Subcultures were made from older colonies by transferring all or portions of the colonies to new medium. A thin film (2-4 mm) of medium (the medium solution without agar) was maintained on the surface of the solidified growth medium. As the culture aged, dehydration of the medium was prevented by the addition of liquid medium with a sterile pipette whenever necessary. This procedure was also used in an attempt to prolong life of older and established cultures, or induce them to produce new and better-differentiated growth.

One of the serious drawbacks of growing still cultures in liquid medium is the resultant oxygen starvation that often causes poor growth. For this reason, liquid cultures were aerated by shaking to determine what effect this might have on growth of the rust fungi used in this study. Freshly-inoculated-125 ml flasks of liquid basal medium were clamped securely on a Burrell Wrist Action Shaker. The cultures were shaken continuously (about 150 cycles/minute) for one week, after which they were incubated as still cultures.

### Observation and Measurement

Periodic visual observations of colonies that developed were made to decide the suitability of the media. These observations were supplemented with microscopic examinations to determine the extent and nature of structures, if any, that were formed. Measurement of structures observed were made whenever possible. Growth characteristics including pigmentation, sectoring, and density were noted. Photographs of various structures and stages of growth were taken.

The suitability of a medium for the in vitro culture of the races used was based primarily on the intensity and extent of growth and differentiation observed after a minimum of four weeks incubation. Growth, as used in this paper, refers to the presence of an intact and growing vegetative mass with varying degrees of differentiation. Differentiation included branching and sometimes the production of various structures such as globose cells and other bodies that resembled spore forms of the leaf rust fungus. The final measure of suitability of the medium was the ability to support, for an extended period of time, saprophytic growth of the fungus under study.

### Media Amendments

After repeated inoculation and observation of colonies produced by each race on the four media tested, the most suitable medium (Medium III Modified Czapek's Mineral Salt Medium) henceforth referred to as the basal medium, was de-

terminated and various amendments added with the objective of enhancing saprophytic growth of the leaf rust fungus. The race used in this part of the study was limited to that found to produce the most extensive growth on the selected basal medium. Both solid and liquid forms of the basal medium were used. The liquid form was prepared by omitting as an ingredient, the solidifying agent, agar. The liquid medium was always contained in 125 ml Erlenmeyer flasks to minimize spilling and contamination during incubation. The effects of the different amendments were determined by comparing general growth characteristics on the amended basal medium, with the growth characteristics on basal medium alone.

The following substances were used as medium amendments:

1. Casamino acid. Bushnell and Rajendren (5) supplemented their medium for P. graminis f. sp. tritici with this compound and noted the formation of unusually large masses of spores. In this study, Bacto Casamino acid, "Difco" Certified, was first dissolved in glass-distilled water and then filter-sterilized through an ultra fine Vacuum Type Morton Bacteriological filter apparatus with a pyrex fritted disc having a nominal maximum pore diameter of 0.9 to 1.4 microns. The sterile solution was aseptically pipetted into a previously sterilized basal medium cooled to about 45 C to yield a final concentration of 0.4 % casamino acid.

2. Zinc as ZnSO<sub>4</sub>. The effect of zinc on biological systems is widely known. Results of studies by Couey and Smith

(9), Sharp and Smith (19) and Sharp (18), indicated that zinc promoted germ tube growth and differentiation in artificial substrate. In the present study zinc was added to the basal medium to yield a final concentration of 10 ppm. This concentration was within the range that Sharp and Smith (19) found to be optimum for vesicle formation by P. coronata avenae Corda.

3. Bovine Serum Albumin (BSA). The BSA used in this study was a type F, essentially fatty acid-free Bovine Albumin, obtained from Sigma Chemical Company, St. Louis, Mo. It was first dissolved in glass-distilled water and filter-sterilized as described above. The sterile filtrate was aseptically pipetted into the basal medium to yield a final concentration of 1.0% BSA.

4. Extract from rust-flecked wheat leaves. Wheat leaves of the cultivar Cheyenne, inoculated with the desired race were detached seven days after inoculation. At this stage the uredosori were almost ready to break the leaf epidermis. The detached leaves were suspended in sterile-distilled water to prevent loss of turgidity while they were brought into the laboratory. There they were further cut into smaller sections about four to six mm long. Five gm of this material was homogenized in a Servall Omni-Mixer for four minutes followed by centrifugation in a Sorvall-SS-3 Automatic Superspeed Centrifuge for 20 minutes at 270 g. The supernatant was then filter-sterilized as described before and pipetted into the basal medium to yield a final concentration

of 1.0% extract.

### Pathogenicity Tests

To determine whether cultures produced on artificial media were capable of infecting wheat, they were inoculated on a susceptible cultivar, Cheyenne. Three methods of inoculation were used. The first two methods were those employed by Williams et. al. (24) in demonstrating the pathogenicity of a stem rust fungus grown in an artificial medium. The first method consisted of cutting the abaxial epidermis with a sharp razor blade. This was stripped away from the mesophyll cell layer five to ten cm with a flat-tipped forceps. A small amount of growth produced on an artificial medium was placed on the exposed mesophyll. The epidermis was laid back in place and the tip of the inoculated leaf covered with Scotch Brand pressure sensitive tape to keep the stripped epidermis intact.

The second method was similar to the first except that whole leaves which were previously surface-sterilized, and with the lower epidermis removed, were laid over cultures with the exposed mesophyll in contact with the culture growing on artificial media.

The third method used whole leaves not detached from the plant. First, the epidermis was stripped back and allowed to remain in a water suspension of the culture growth taken from artificial media for about 72 hours. These leaves were withdrawn from the suspension and the epidermis laid

back in place and held secure with Scotch Brand pressure sensitive tape.

All plants or detached leaves were kept in a saturated atmosphere for 24 hours after inoculation and then transferred to a growth chamber (Percival Model E-30) with a 12-hour daily lighting (5381.95 lux) at  $17 \pm 1$  C for further observation.

## CHAPTER IV

### RESULTS

#### Growth on Basal Media

After examining a great many cultures it became apparent that each of the media would support varying degrees of growth of the leaf rust fungus. Descriptions of growth characteristics of the fungus on each medium used are presented below. Unless indicated, these descriptions apply to all races on that medium.

Medium I. After two to three days incubation, growth appeared as an off-white mycelial mat that became increasingly profuse and slightly darker in color with age. Under the microscope the growth appeared as a dense mass of very long, slender and unbranched hyphae (Figure 1). After 10 to 13 days incubation, the mycelial mat assumed a dull white to light brown color. Three to seven days later it became brown and appeared as a loose vegetative mass of moist and shrivelled hyphae. Subcultures invariably lost turgor and collapsed after a minimum of ten days incubation. Pipetting fresh liquid medium into older intact cultures failed to induce formation of new growth. This treatment, however, delayed the collapse of the vegetative mass for a period ranging from two to four days.

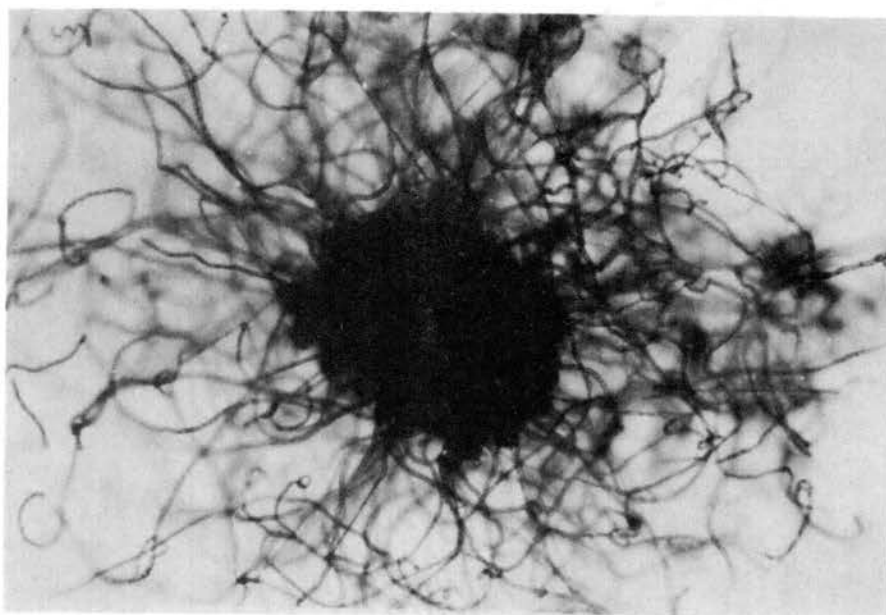


Figure 1. Dense mass of slender, unbranched hyphae of Puccinia recondita f. sp. tritici race UN 1A on Medium I (PDA) after three days. 82X

Medium II. Early growth on this medium consisted of a scanty, thin layer of pale brown mycelium dispersed at the surface of the medium. Further incubation did not result in any appreciable amount of additional growth. It was observed however that a closer contact of the mycelial mat and the medium was established. Microscopic examination revealed the presence of branched, sporophore-like hyphae with seemingly rigid walls (Figure 2). This kind of growth was generally observed after four to six weeks incubation and remained intact for two to several weeks, depending upon the condition of the medium. Sometimes, especially among cultures of races UN 1A and UN 2A, tips of branched, sporophore-like hyphal strands formed chains of globose cells with diameters ranging from 4.1 to 7.6  $\mu$ .

The well-differentiated saprophytic growth was observed from cultures of all races except race UN 9. It should be pointed out, however, that the races that did produce growth on this medium did not always do so every time attempts to grow them axenically were made. For instance, well-differentiated growth was observed with about 20% of the cultures of races UN 1A, UN 2A and UN 2AAG. About five to ten per cent of the cultures of races UN 2, UN 5A and UN 13 produced growth on this medium and cultures of race UN 6B hardly grew at all.

Subcultures prepared by seeding six to eight-week old cultures to fresh medium were not very successful. The main difference between cultures that were allowed to remain on

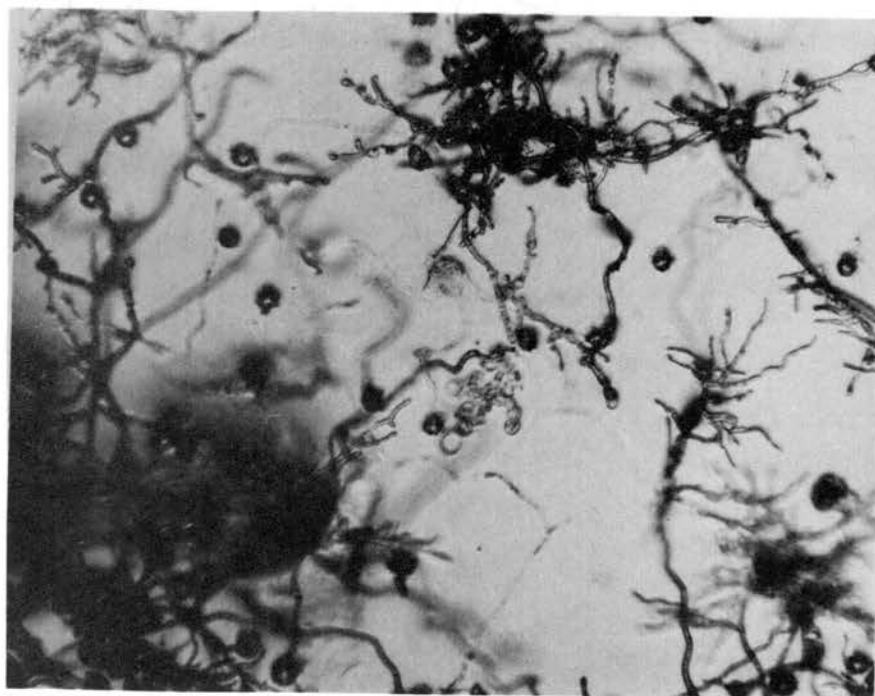


Figure 2. Branched, rigid-appearing sporophore-like hyphae produced by Puccinia recondita f. sp. tritici race UN 5A on Medium II (Modified Czapek's Dox Broth Medium), after 36 days. Note chain of globose cells at the tips of some sporophore-like hyphae. 82X

the old medium, and the subcultures on fresh medium was the delayed collapse of the growth mass on those transferred to fresh medium. Subcultures from races UN 2, UN 6B and UN 13 persisted for an additional period of one to two weeks before collapse occurred while subcultures of races UN 1A, UN 2A, UN 2AAG and UN 5A were found to persist much longer ranging to four weeks or more.

When fresh liquid medium was pipetted into established cultures where the medium showed signs of dehydration, growth remained intact for a considerably longer time than when the medium was allowed to continue to dessicate. In a few instances, cultures of races UN 1A and UN 2A produced new growths consisting of a thin layer of mycelial strands, spreading out from the established colony.

In one study involving ten isolates of race UN 2A, one isolate of the ten had distinctly different growth than the others. Instead of branched, rigid-looking hyphal strands, this particular isolate had a profusely branched vegetative mass (Figure 3). Branching arose along practically the entire length of the main hyphal strands. Closer examination revealed that the older branchlets terminated into chains of elongated and club-shaped cells. After about two weeks further incubation, dense and relatively thick-walled cells arising in chains from a common origin were clearly visible (Figure 4). Two to four cells were present in each chain. It appeared that individual cells gradually became globose as they matured. The terminal cell, which was the oldest



Figure 3. Profusely-branched vegetative hyphal mass with older branchlets terminating in chains of elongated and club-shaped cells produced by Puccinia recondita f. sp. tritici race UN 2A after 29 days on Medium II (Modified Czapek's Dox Broth Medium), 82X

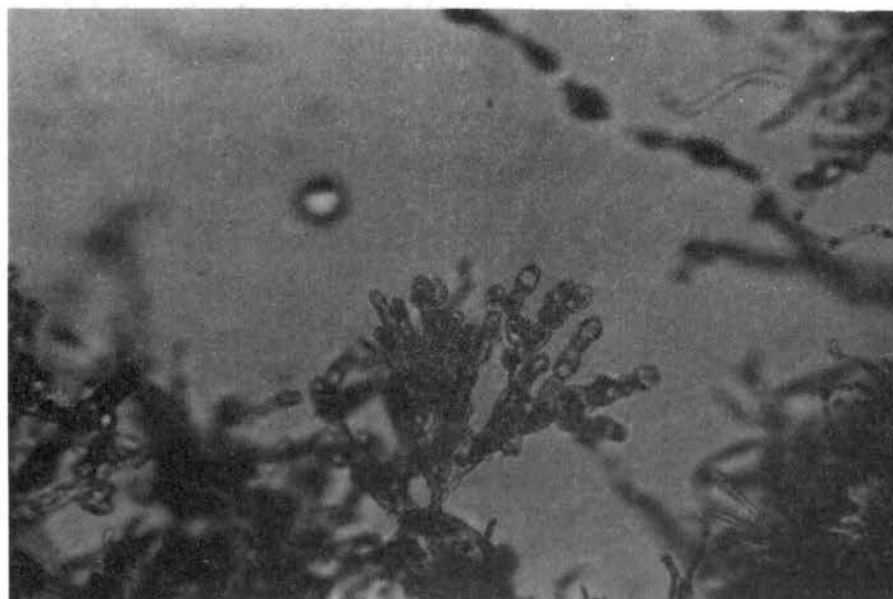


Figure 4. Globose cells in chain with relatively thick walls arising from a main sporophore produced by Puccinia recondita f. sp. tritici race UN 2A after 37 days on Medium II (Modified Czapek's Dox Broth Medium). 140X

became globose first followed by the next oldest just below it. After another two weeks practically all of these cells had become spherical in shape with dense cytoplasm and relatively thick walls (Figure 5). Measurement of 50 cells at this time showed that their diameters ranged from 9.4 to 13.6  $\mu$ .

Medium III. Early growth on this medium was similar to that on Medium II. Starting about the fourth week of incubation, irregularly-shaped and dense mycelial mats in very close contact with the medium, were observed. Growth appeared as being partly buried into the medium. As with Medium II, however, these dense growths were not formed with every attempt to obtain axenic cultures of the fungus, but all races used except race UN 9 did produce this type of growth. Microscopic examination revealed this consisted of clusters of rigid-looking, branched, sporophore-like structures with distinct septations (Figure 6). This kind of growth remained intact for several weeks without any conspicuous change such as the shrivelling or discoloration which was often associated with starved or staling cultures. Neither was there any conspicuous indication of additional growth even after six to eight weeks of further incubation. This behaviour was particularly obvious among cultures of races UN 2 and UN 13, but with certain other races (UN 1A, UN 2A and UN 2AAG), a slight swelling of the terminal cell was observed.

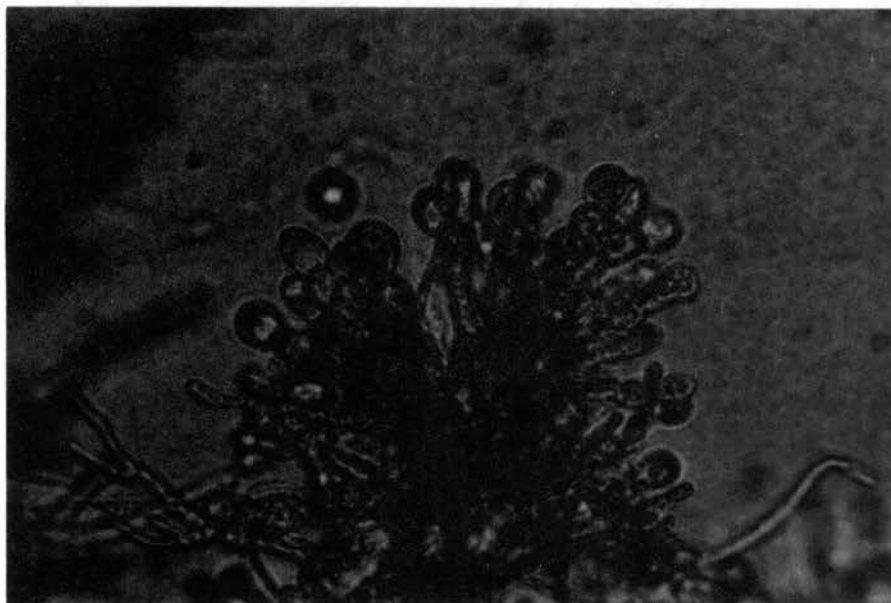


Figure 5. Clusters of globose cells most of them having thick walls and dense cytoplasm produced by Puccinia recondita f. sp. tritici after 47 days on Medium II (Modified Czapek's Dox Broth Medium). 275X

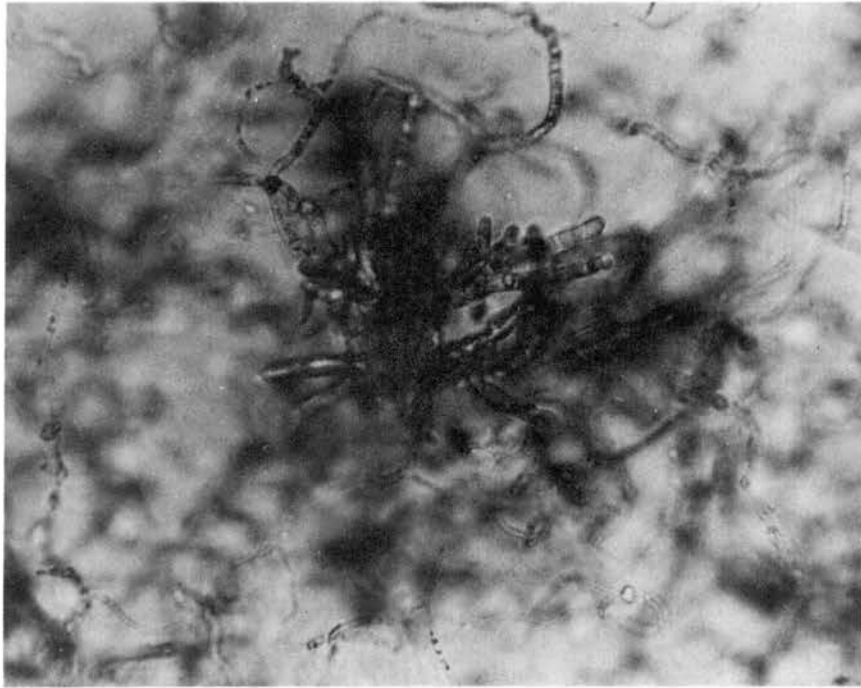


Figure 6. Rigid-looking, branched sporophore-like growth with distinct septations produced by Puccinia recondita f. sp. tritici race UN 2AAG after 37 days on Medium III (Modified Czapek's Mineral Salt Medium). 275X

When fresh medium was pipetted into older established cultures, interesting developments were noted. Cultures of races UN 1A, UN 2A, UN 2AAG, UN 5A and UN 13, particularly, exhibited new growth around the original dense mycelial mats. At first this was manifested as a thin layer of off-white mycelial threads which spread slowly on the growth-free portion of the medium. From the central portion of the original dense regions, masses of stromatic mycelia were formed which finally developed into orange, almost spherical stroma-like bodies. The new surrounding vegetative mass continued to grow thicker until new dense mats were formed which also gave rise to additional stroma-like bodies. Soon, several of the individual stroma-like bodies joined together and formed a large irregularly-shaped stroma-like structure (Figure 7). With age, the original dense mat turned brown and finally black. By this time one or more stroma-like regions had formed around it (Figure 8).

Dense mycelial regions that did not develop into stroma-like structures did exhibit some hyphal growth. The younger strands appeared to be septate, branched and relatively slender, but dense and rigid-looking. The older ones showed profuse branching at certain regions along their length. Highly differentiated growth was evident in these regions, being exhibited as a chain of globose or other variously shaped cells (Figure 9).

In the beginning it was thought that individual stroma-like bodies were smooth-sided compact vegetative structures.

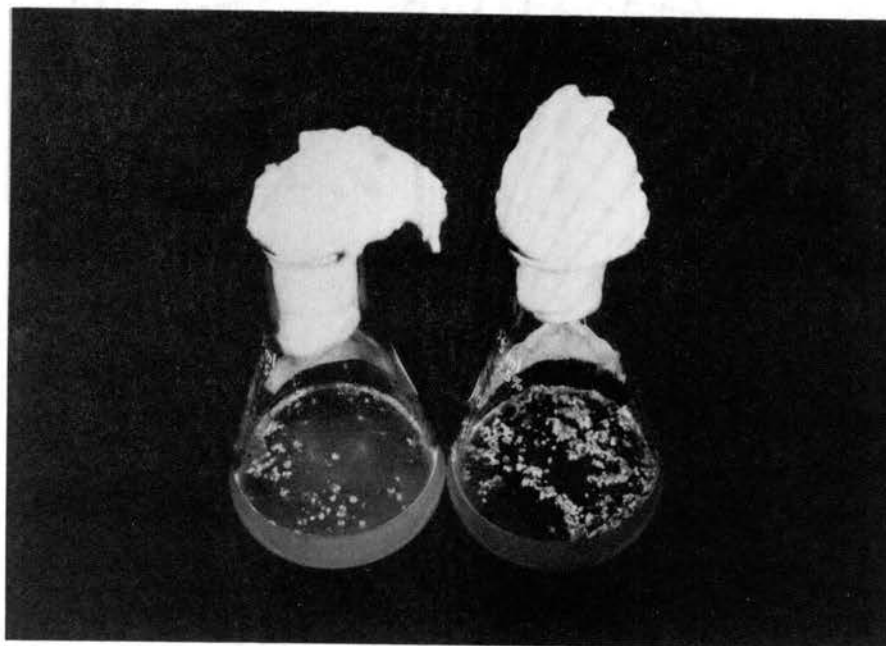


Figure 7. Irregularly-shaped clusters of stromatic-like growth of Puccinia recondita f. sp. tritici race UN 2AAG after 30 days (left) and 47 days (right) on the solid form of Medium III (Modified Czapek's Mineral Salt Medium). 0.25X

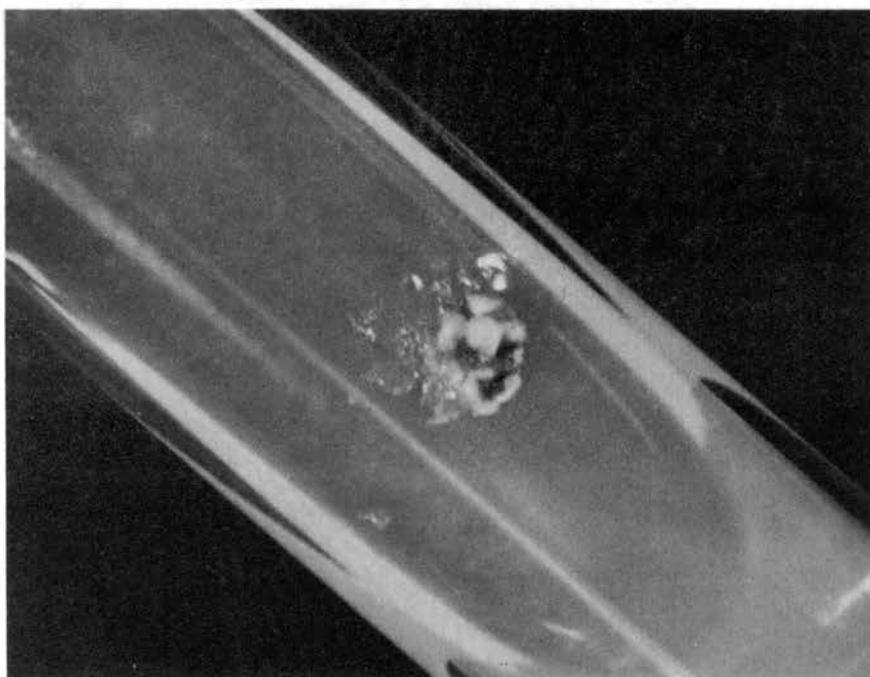


Figure 8. New stroma-like regions around an original stroma-like structure that has started to turn brown. Puccinia recondita f. sp. tritici race UN 2A after 48 days on Medium III (Modified Czapek's Mineral Salt Medium). 2.5X

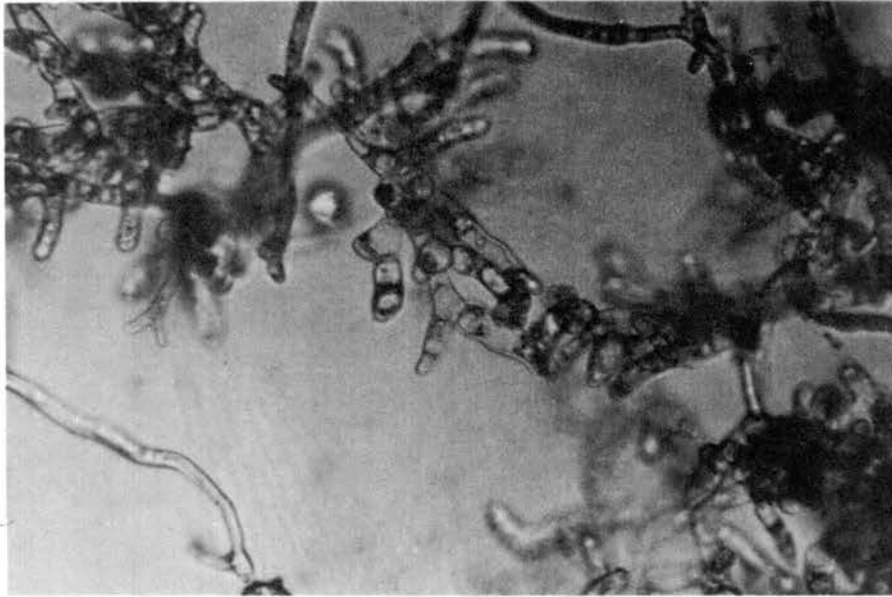


Figure 9. Hyphal strands in various stages of development. Younger hyphae are slender with few branches; older ones show globose cell formation. Puccinia recondita f. sp. tritici race UN 1A after 48 days on Medium III (Modified Czapek's Mineral Salt Medium). 275X

However, closer microscopic examination revealed various types of differentiation on the sides of these bodies. Narrow, septate and rigid-looking hyphal threads were observed first. With further incubation, these developed into chains of elongate cells which finally became globose. After reaching this stage, no further differentiation was observed with races UN 1A, UN 2AAG, UN 5A and UN 13. The globose bodies they produced were markedly underdeveloped and immature, particularly with races UN 1A and UN 13 (Figure 10). The thin-walled globose cells developed no further and one to two weeks after they were formed they collapsed. At this stage, discoloration of the stromata from orange to brown took place.

Approximately 40% of cultures of race UN 2A produced further differentiation of the globose cells found protruding from the sides of the stromata. Some of them had become decidedly more dense, while others appeared to be solid, almost spherical bodies (Figure 11). An increase in diameter also occurred which finally produced cells ranging from 10.6 to 13.9  $\mu$ .

As with Medium II, subcultures made by transferring growth to new medium, remained intact for a longer time than when growth was allowed to remain on the original medium. When incubated for a period from four to six weeks, new stroma developed on subcultures of races UN 2A, UN 2AAG and UN 5A, but this was considerably longer than was required to produce new growth when medium was simply pipetted into the

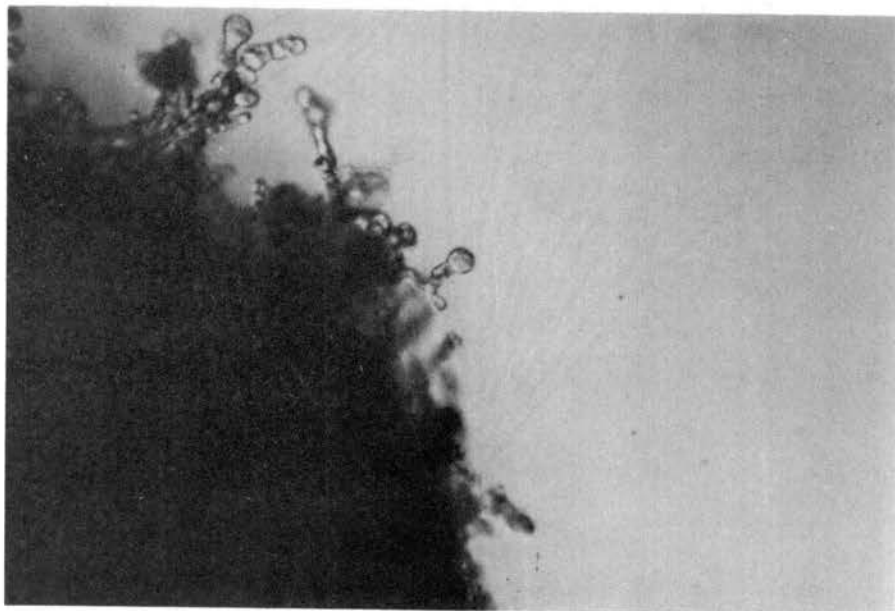


Figure 10. Stromatic-like growth with globose cells protruding from the sides produced by Puccinia recondita f. sp. tritici after 44 days on Medium III (Modified Czapek's Mineral Salt Medium). 140X. Note thin walls and immature appearance.

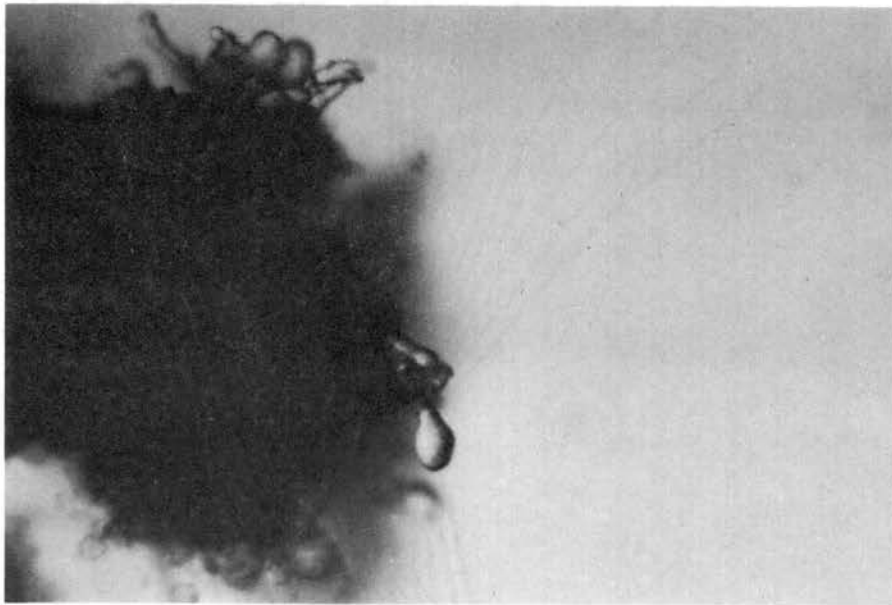


Figure 11. Dense, solid-looking globose cells on the sides of stromatic-like growth produced by Puccinia recondita f. sp. tritici race UN 2A on Medium III (Modified Czapek's Mineral Salt Medium). 140X

original culture vessel.

Additional study revealed that clusters of stromatic growth were more consistently produced on cultures of races UN 2A, UN 2AAG and UN 5A than with the other races. This was especially so when a film of medium (in liquid form) was maintained on the surface of the solid growth medium.

Certain cultures of race UN 2A produced uredospore-like bodies never observed in cultures of any other race used in this study. The sporophores were thickly massed together and appeared as hyphal threads with evident septations. The uredospore-like bodies were borne at the tip of individual sporophores (Figure 12). Closer examination revealed that these bodies had verrucose walls characteristic of uredospores produced in living hosts. Measurement of uredospore-like bodies thus far observed showed that they had an average diameter of  $14.4\ \mu$ . Fresh uredospores from wheat plants were found to have an average diameter of  $16.2\ \mu$ .

Cultures of race UN 2A have persisted longer than any of the other races tested. Thus far, one isolate has survived five subculture transfers made at intervals of 50 to 60 days. New growth of subcultures has always appeared first as dull white mycelia ultimately turning yellowish and then forming an orange stroma. These always developed on the new medium around the old, transferred stromata. Microscopic examination of the sides of the old stromata invariably showed clusters of sporophores that ultimately gave rise to globose and other variously shaped cells. When the old

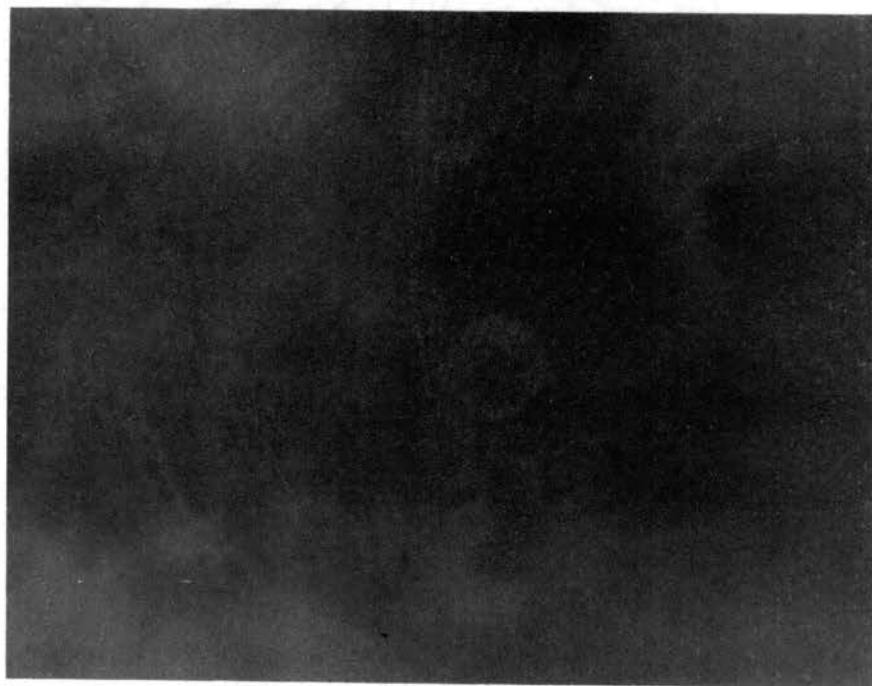


Figure 12. Uredospore-like body at the tip of a sporophore of Puccinia recondita f. sp. tritici race UN 2A after 63 days on Medium III (Modified Czapek's Mineral Salt Medium). 275X

stromata were squashed and examined under the microscope abundant uredospore-like bodies were always found.

Medium IV. A very characteristic growth was produced on this medium. At first, a relatively thin mycelial mat was observed at about the sixth day of incubation. This increased in density during the next six to ten days, until it developed into a very profuse white to light tan vegetative mat. Microscopic examination revealed this growth to be distinctly different from that formed on other media. The mycelial threads appeared distinctly shrivelled. Septa were present but not distinct due to the obliterated outline of the mycelial threads (Figure 13). Another distinctive characteristic was the presence of thickened regions along the length of an individual hypha (Figure 14). These structures appeared to be most commonly formed in cultures of races UN 2AAG and UN 5A. In spite of the shrivelled appearance of the vegetative growth, cultures on this medium persisted for a relatively long time even without subculturing. Cultures of races UN 1A, UN 2A, UN 2AAG and UN 5A were found to remain intact for eight weeks or more when fresh medium was pipetted into older cultures with intact growth.

#### Effects of Media Amendments on Growth

It was apparent from the results, that of the four media used, Medium III (Modified Czapek's Mineral Salt Medium) was the most suitable in supporting saprophytic growth of the leaf rust fungus. Also, of the eight races, UN 2A was

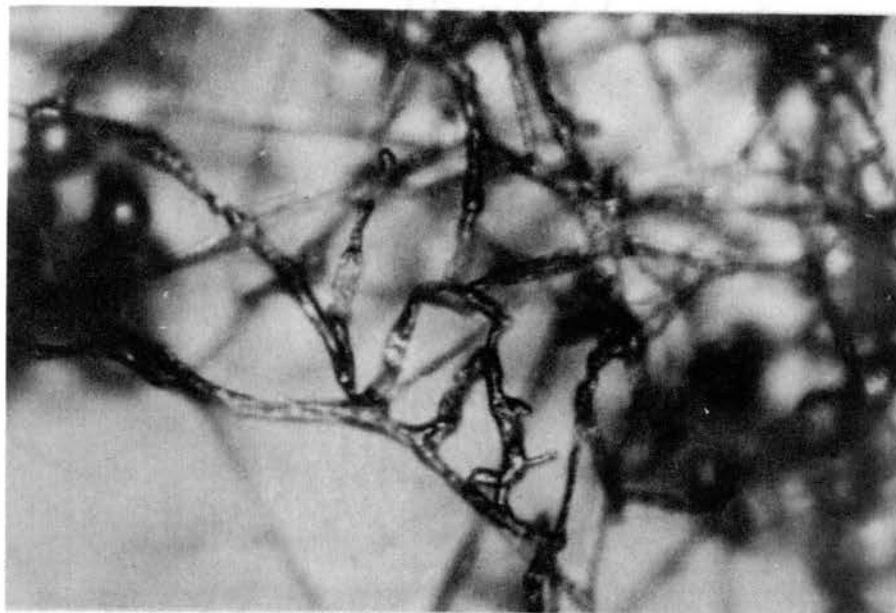


Figure 13. Mycelial strands of Puccinia recondita f. sp. tritici race UN 2AAG after 20 days on Medium IV (Trace Element Medium). 140X. Note shrivelled appearance.

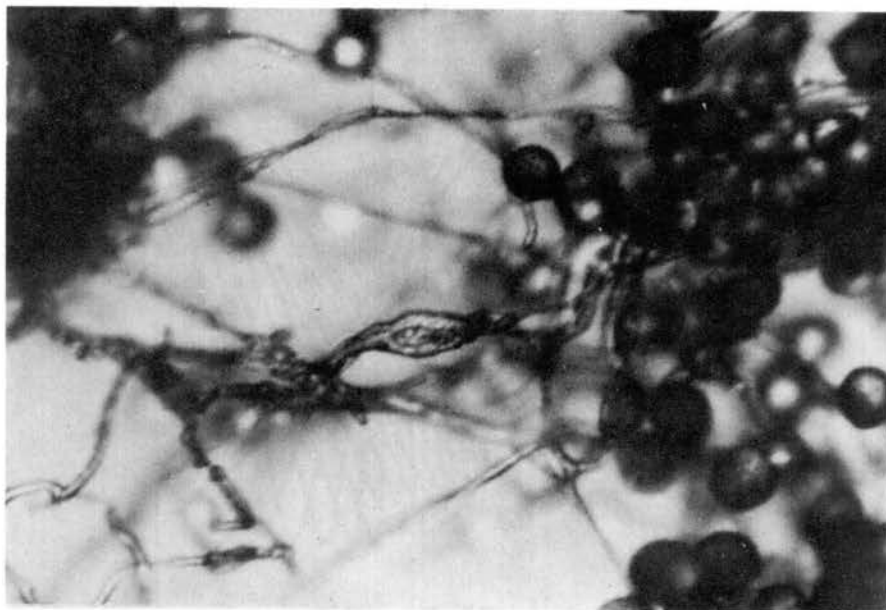


Figure 14. Puccinia recondita f. sp. tritici race UN 2AAG. Mycelial strands with thickened cell after 56 days on Medium IV (Trace Element Medium). Uredospores are part of original inoculum. 140X

superior to the other races in persistence of growth as well as the ability to produce differentiated structures including uredospore-like bodies, that were found on cultures of this race growing on Medium III.

Using race 2A, various amendments were added to Medium III to see if further growth or differentiation could be induced. Both solid and liquid forms of the medium were used.

Solid Medium. On Medium III alone the characteristic colony consisted of a thin layer of light brown vegetative growth which later developed into dense regions that gave rise to stromata. When Sigma Bovine Serum Albumin (BSA) was added and the medium inoculated with uredospore dispersed in 15% gelatin, modification of colony appearance was observed. Instead of a thin layer of vegetative mass as appeared on Medium III alone, growth on the amended medium consisted of a layer of vegetative mass with prominent aerial growth. Under the microscope this aerial growth appeared as groups of slender mycelial strands with moderate branching (Figure 15). Older colonies on the amended medium had structures similar to those that this race characteristically produced on Medium III alone. These consisted of thick-walled and rigid-looking hyphal strands which differentiated into chains of globose and other variously shaped cells. Stromata formation however, was noted to be considerably reduced on the amended medium. More often, the stromata appeared as solitary units scattered on the surface of the medium.

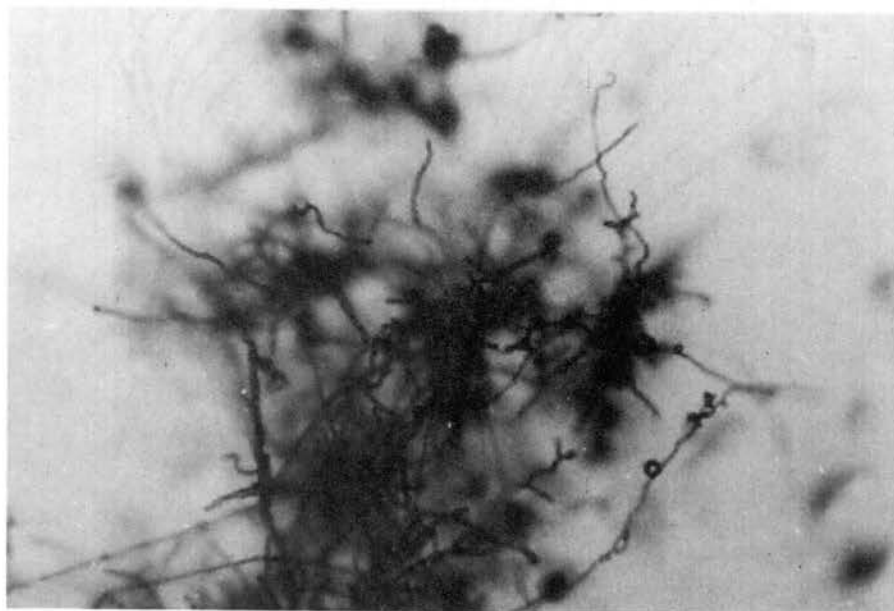


Figure 15. Aerial growth after 23 days consisting of moderately branched, slender mycelial threads produced on Medium III amended with 1% Sigma Bovine Serum Albumin and seeded with uredospores of Puccinia recondita f. sp tritici race UN 2A dispersed in 15% gelatin. 82X

Compared with growth on Medium III alone, no conspicuous change in growth appearance was noted when the medium was amended with casamino acid,  $\text{ZnSO}_4$  or extract from rust-flecked wheat leaves. In general, the extent of differentiation, including the formation of globose and other variously shaped cells, and the production of aggregated stromatic bodies was similar to that found on the basal medium alone.

Liquid Medium. Growth of still liquid cultures was first manifested as a thin film of light brown vegetative mat after two weeks incubation. Dense regions were not visible until the fifth to sixth week of incubation, which was one to two weeks later than on solid medium. Each dense region formed into a solitary orange stroma body. Later, these solitary stromata tended to aggregate to form enlarged and irregularly-shaped clusters of stromatic bodies (Figure 16).

Growth of shake cultures consisted of a very thin, light brown, slimy mycelial mass. It was usually found submerged or adhering to the wall of the culture vessel. Growth differentiation was limited to a few hyphal strands with branch initials. No dense colony, much less stromatic growth, was produced.

No obvious difference was noted in growth characteristics of cultures on Medium III alone compared with those on the same medium amended with casamino acid,  $\text{ZnSO}_4$  and extract from rust-flecked wheat leaves. With or without amendment, shake cultures were considerably limited in growth compared with still cultures.

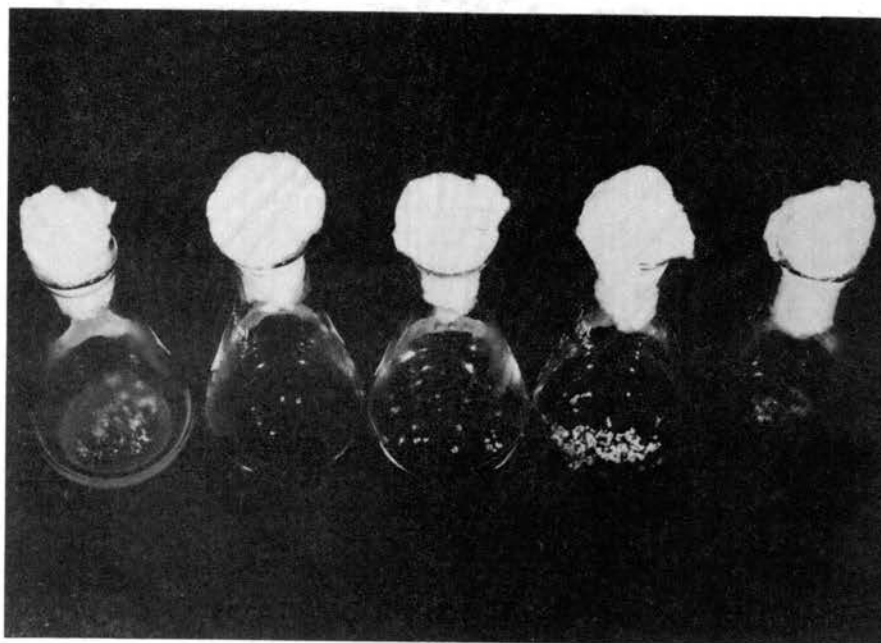


Figure 16. Stages of stromatic growth of Puccinia recondita f. sp. tritici race UN 2A on liquid Medium III (Modified Czapek's Mineral Salt Medium. Left to right: Early growth showing dense film of mycelia on the surface of the medium; film becomes very thin; appearance of solitary, orange stromatic bodies; fusion of individual stromata to form irregularly-shaped clusters of stromatic growth; old, discolored and compact stromatic growth. 0.25X

### Growth of the Stem Rust Fungus on Medium III

On Medium III four-to six- week old cultures of the stem rust fungus, P. graminis f. sp. tritici Australian race 126, ANZ 6,7, showed dense regions of vegetative growth. Branched, rigid-looking hyphal strands were quite evident with some branches terminating in variously shaped bodies, including structures that resembled rudimentary spores (Figure 17). Stromatic growth was noted on seven-week old cultures and microscopic examination revealed globose and other variously shaped structures protruding from the sides of these aggregated stromata (Figure 18). No attempt was made to grow the fungus on any of the other media used in the study.

### Test of Pathogenicity of Cultures

All of the attempts to infect wheat by inoculating it with various types of growth formed on artificial medium failed. When inoculated by the sub-epidermal technique and incubated for seven days the only change observed was a discoloration of the leaf tissue from green to light brown, around the point where the inoculum was introduced (Figure 19). No attempt was made to determine microscopically whether the fungus had started new growth on the host, but macroscopically there was no sign of any type of growth or fructification, even after incubating the inoculated leaves for more than two weeks. The other methods of inocu-

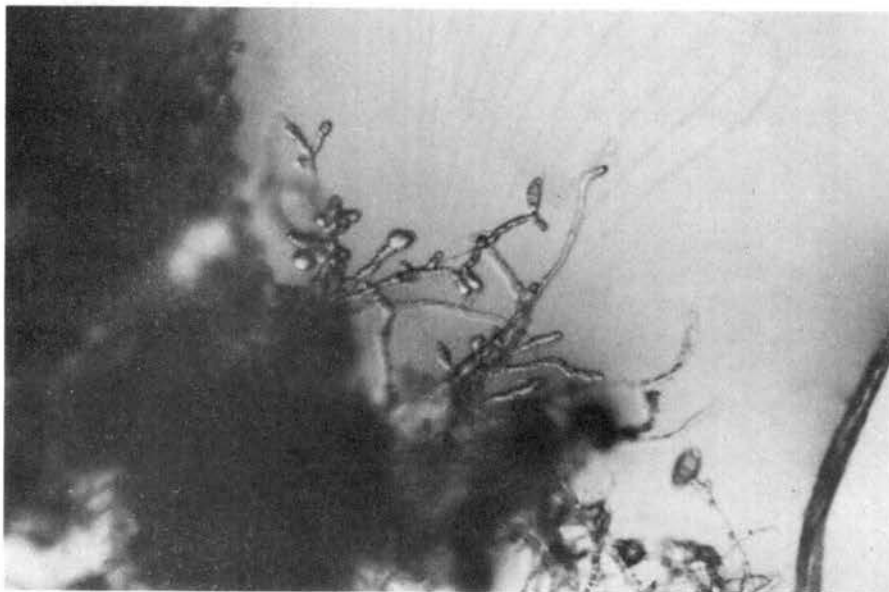


Figure 17. Rudimentary spores produced by a 43-day old culture of Puccinia graminis f. sp. tritici, Australian race 126 ANZ 6,7 on Medium III (Modified Czapek's Mineral Salt Medium). 140X

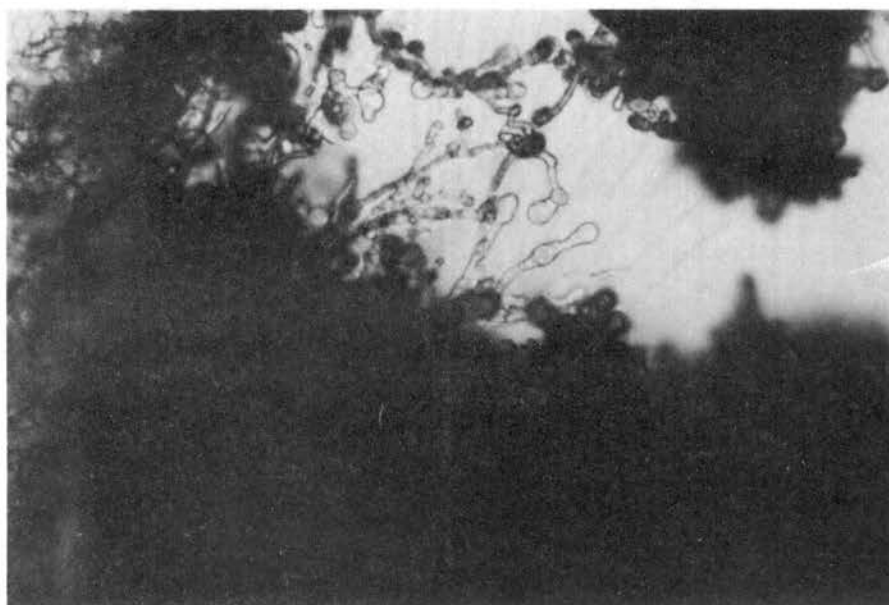


Figure 18. Variously-shaped structures on the sides of stromatic growth of Puccinia graminis f. sp. tritici, Australian race 126 ANZ 6,7 on Medium III (Modified Czapek's Mineral Salt Medium) after 51 days. 140X



Figure 19. Cheyenne wheat leaf inoculated with a portion of the growth of Puccinia recondita f. sp. tritici race UN 2A formed on Medium III (Modified Czapek's Mineral Salt Medium). Note the discoloration of tissues around the inoculum (dark area), 20X

lation used did not result in any visible infection. Tissues that were in contact with the inoculum did not exhibit any discoloration.

## CHAPTER V

### DISCUSSION

The results show that the leaf rust fungus can be grown on artificial media, and confirm the work of Singleton and Young (20). Marked differences in the amount of growth and the extent to which the fungus differentiated on the media tested certainly suggests that the problem involved in growing the fungus axenically is primarily nutritional. This opinion is consistent with that of Scott and Maclean (17) who wrote that if growth of the rust fungus in culture were to occur, a major variable to contend with would be the selection of nutrients. This opinion was supported by Cutter (10) who found that the addition of certain ingredients to a basal medium induced much better growth of saprophytic cultures of G. juniperi-virginianae. Similarly, Singleton and Young (20) found that the leaf rust fungus exhibited greater differentiation on certain specialized media compared with water agar alone.

Examination of colony characteristics on each of the media tested showed that the kinds of growth can be conveniently classified into three types of response of uredospore-seeded cultures of the rust fungi as described by Scott and Maclean (17). The three types are : (a) germination oc-

cured but saprophytic growth did not initiate even after extended periods of incubation, (b) vigorous saprophytic growth occurred leading to formation of a dense, white hyphal mat on the surface two to three weeks after seeding and (c) germinated uredospores exhibited saprophytic growth within the first two to three weeks, and with further incubation mycelial tufts or stromatic growth became macroscopically visible 30 to 60 days after seeding.

In these experiments the first type would be exemplified by growth on Medium I (PDA). On this medium germ tubes elongated into an extensive vegetative mass of mycelia, but this soon began to collapse and death set in. The marked inferiority of PDA as growth medium may be due to a low level or imbalance of required minerals, or other substances. Sustainance for the dense vegetative mass which occurred early in the incubation period probably came from endogenous substances in the spore inoculum. When these food reserves were exhausted, growth stopped as indicated by the collapse and discoloration of the vegetative mass after ten or more days of incubation.

Growth on Medium IV (Trace Element Medium) may be classified as the second type, where saprophytic growth consisted of a dense vegetative mat without any clear indication of sporulation. The vegetative growth on this medium was even more persistent than that described by Scott and Maclean (17). Growth of some races remained intact not just for two to three weeks but as long as eight weeks without sta-

ling. The absence of sporulation suggests that the medium was short of certain components which would enable the fungus to reach this stage of growth.

Growth on Medium III (Modified Czapek's Mineral Salt Medium), and to some extent Medium II (Modified Czapek's Dox Broth Medium) fit into the third type. As earlier described, growth on these media consisted, in the beginning of the incubation period, of scanty mycelial growth which gave the appearance of being partly buried in the medium. On Medium III, this growth slowly gave rise to dense regions that ultimately formed clusters of orange stromatic growth. Stromatic growth has been observed to be consistently associated with what are now considered as rudimentary spore forms of the fungus. The ability of Medium III to support the production of these spore forms, and also, to a limited extent, nearly normal uredospores, make it a very promising medium and deserves further investigation.

The association of spore-like bodies with stromatic growth has been reported by various other investigators. Bushnell (4) observed that hyphae produced by the stem rust fungus in growth media, were superseded by more nearly globose cells at three to six weeks after seeding, and growth centers combined to form one or more compact stromata within each seeded zone. Turel (21) reported spherical uredospore-like structures from compact masses of small cells that developed in cultures of the flax rust fungus. She added that the appearance of the spores suggested that they were

not quite mature.

The superiority of Medium III (Modified Czapek's Mineral Salt Medium) for supporting growth over the other media tested, suggests the usefulness of the ingredients relative to the needs of the rust fungus for saprophytic existence. Perhaps even more important was the relative balance of the ingredients that were present in the medium. In her work on the saprophytic development of the flax rust fungus, Turel (21) reported that growth was observed on a medium containing Difco yeast extract, sucrose and inorganic salts. However, when Czapek's minerals differing both in type and quantity were included as source of inorganic constituents, no growth was obtained. She concluded that the balance of inorganic salts played an important role in determining growth of the fungus. It is apparent, however, that Medium III is still deficient in some form since it failed to provide the fungus sufficiently well to permit normal sporulation. In this study, structures that resembled nearly normal uredospores were formed but only in very meager quantity. Rudimentary spore-like bodies were formed more abundantly, but even after prolonged incubation did not undergo further differentiation or maturation.

Differences in culturability of the races was evident from variations in the extent and persistence of growth in each of the media. Even on the best medium (III) only six (UN 1A, UN 2, UN 2A, UN 2AAG, UN 5A and UN 13) out of the eight original races showed distinct development beyond the

germ tube stage. Only five of these formed stromatic growth on which were found rudimentary spore forms of the fungus, and only one race (UN 2A) showed further differentiation to the extent of nearly normal uredospores. And it was only this one race that continued to produce stromatic growth after five subculturings at 50-to-60 day intervals.

It is believed that these marked differences reflect the ability of the races to adapt to saprophytic growth. Whether a race was adapted to a medium or not depended upon the genetic constitution of the race, the nutritional composition of the medium and the conditions provided during incubation. It has been suggested that saprophytic growth of the rust fungi depends upon the ability of the organism to effect a change in genome (17). This ability would be determined by the nutritional environment to which they were exposed, together with the composition of the cytoplasm, which would provide, for example, carry-over nutrients. It was further suggested that genetic variation within species was also important. Coffey et. al. (8) stated that differences in growth rate between two strains of M. lini when inoculated to artificial medium could have been due to genetic or physiological factors or both. Bushnell and Stewart (6) concluded that the large differences they observed in growth and development of American isolates of the stem rust fungus were due to genetic effects. Williams et. al. (23) stated that growth differences between races of rust fungi in response to growth conditions, may be a reflection of the

amount of endogenous growth substances, genetic constitution of individuals, or the influence of various physiological factors.

None of the amendments to Medium III promoted further growth of the fungus. Modification of colony characteristics from thin vegetative mats to dense colonies with aerial growth was noted when 1% BSA was added to the basal medium, and the uredospore inoculum was implanted using a 15% gelatin as a suspension medium. In their work on the in vitro culture of the flax rust fungus, Coffey et. al. (7) observed a distinct characteristic growth mass, when they supplemented their medium with BSA, and seeded their uredospores using a gelatin suspension. They described growth as in the form of masses of fluffy white aerial mycelia. Apparently the effect was similar with cultures of P. recondita f. sp. tritici.

Better growth and differentiation of the leaf rust fungus was obtained in this study than has been previously reported. Improved inoculating technique (allowing the spore to drop freely to the surface of the medium), and the practice of maintaining a film of liquid (medium without agar) on the surface of the solidified growth medium, resulted in considerably improved growth. Rehydrating medium of older established cultures favored additional mycelial formation and further growth differentiation. It is believed that improved growth was also facilitated by better race-medium combinations, adequate supplies of aseptic spores for inoc-

ulum, and improved aeration through the use of cotton-plugged flasks as culture vessels.

## CHAPTER VI

### SUMMARY

1. Four media: PDA, Modified Czapek's Dox Broth, Modified Czapek's Mineral Salt and Trace Element Media, were tested to determine the most suitable one for growth of the wheat leaf rust fungus, Puccinia recondita f. sp. tritici.
2. PDA did not support saprophytic growth. Growth consisted of elongated and extensive vegetative mass which became shrivelled after ten or more days.
3. Growth on Modified Czapek's Dox Broth appeared as thin layers of mycelium with branched, sporophore-like hyphae that sometimes gave rise to globose cells.
4. Modified Czapek's Mineral Salt Medium supported dense mycelial growth which formed into clusters of orange stromata bearing rudimentary and sometimes nearly normal uredospores. This was the best of the four media tested.
5. Growth on Trace Element Medium appeared as dense, non-sporulating vegetative masses that remained intact for a considerably long time.
6. When 1% Sigma Bovine Serum Albumin (BSA) was added to the basal medium and the inoculum implanted using a 15% gelatin as suspension medium, early growth was modified

from thin vegetative mats to dense colonies with aerial growth.

7. Eight races of the fungus (UN 1A, UN 2, UN 2A, UN 2AAG, UN 5A, UN 6B, UN 9 and UN 13) differed in their ability to grow saprophytically. Six of the eight races (UN 1A, UN 2, UN 2A, UN 2AAG, UN 5A and UN 13) developed beyond the germ tube stage; five races (UN 1A, UN 2A, UN 2AAG, UN 5A and UN 13) produced stromatic growth with globose cells resembling rudimentary uredospores; one race, UN 2A, produced nearly normal uredospores. The same race continued to grow after five subculture transfers at 50- to 60-day intervals.
8. The most differentiated and persistent growth was obtained with the combination of cultures of race UN 2A inoculated on Medium III (Modified Czapek's Mineral Salt Medium). Considerable differentiation was obtained with combinations of UN 2AAG-Medium III, UN 5A-Medium III and to a much lesser extent UN 2A-Medium II and UN 1A-Medium II combinations.

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## APPENDIX

Composition of media used for in vitro culture of Puccinia recondita f. sp. tritici.

### Medium I (Potato Dextrose Agar)

20g Dextrose  
15g Agar  
Decoction from 200g  
peeled, diced and  
boiled potato  
Glass-distilled water  
- to 1 liter

### Medium II (Modified Czapek's Dox Broth Medium)

35g Czapek's Dox Broth  
1g Peptone (Evan's)  
1g Yeast Extract  
20g Agar (Bacto-Difco)  
Glass-distilled water  
- to 1 liter

### Trace Element Solution Added to Medium IV

10mg H<sub>3</sub>PO<sub>4</sub>  
13g Sequestrene (13% NaFe)  
20mg BeSO<sub>4</sub>  
447mg MnSO<sub>4</sub>  
10mg KI  
18mg NiCl<sub>2</sub> · 6H<sub>2</sub>O  
18mg CoCl<sub>2</sub> · 6H<sub>2</sub>O  
42mg Ti(SO<sub>4</sub>)<sub>2</sub> · 9H<sub>2</sub>O  
35mg ZnSO<sub>4</sub>  
15mg CuSO<sub>4</sub> · 5H<sub>2</sub>O  
0.2ml H<sub>2</sub>SO<sub>4</sub> (conc.)  
Glass-distilled water  
- to 0.2 liter

### Medium III (Czapek's Mineral Salt Medium)

2g NaNO<sub>3</sub>  
.5g KCl  
.5g MgSO<sub>4</sub> · 7H<sub>2</sub>O  
1g KH<sub>2</sub>PO<sub>4</sub>  
10mg FeSO<sub>4</sub> · 7H<sub>2</sub>O  
30g Glucose  
4g Peptone (Evan's)  
15g Agar (Bacto-Difco)  
Glass-distilled water  
- to 1 liter

### Medium IV (Trace Element Medium)

1.5g Ca(NO<sub>3</sub>)<sub>2</sub> · 4H<sub>2</sub>O  
250mg KNO<sub>3</sub>  
250mg MgSO<sub>4</sub> · 7H<sub>2</sub>O  
250mg KH<sub>2</sub>PO<sub>4</sub>  
40mg NH<sub>4</sub>NO<sub>3</sub>  
40g Sucrose  
1g Yeast Extract  
500mg K<sub>2</sub>HPO<sub>4</sub>  
.5ml Trace Element  
Solution  
Glass-distilled water  
- to 1 liter

## VITA

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